

SURFACE MULTI-FUNCTIONALIZATION OF POLY(LACTIC ACID) NANOPARTICLES AND C6 GLIOMA CELL TARGETING *in vivo**

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Abstract Polysaccharide coated PLA nanoparticles bearing aldehyde groups were prepared by dialysis of DMSO solution of cholesterol hydrophobic-modified dextran polyaldehyde and PLA against water. The average diameter of the nanoparticles was about 160 nm, and the size distribution was nearly homogenous. The nanoparticles were functionalized simultaneously with CD71 and EGFR antibody through the Schiff's base reaction, and then radiolabeled with ^{99m}Tc. After perfused the radiolabeled nanoparticles into tumor-bearing rats through left common carotid artery, the radioactivity in liver, spleen, kidney and brain was measured by scintillation counter. The results showed that less than 2% of nanoparticles were uptaken by the brain due to the uptake of the nanoparticles by the RES system. However, the coupling of transferrin antibody on the nanoparticles facilitated the penetration of nanoparticles across the blood brain barrier, and more specially, compared with monofunctionalized and native nanoparticles, the multifunctionalization enhanced the tumor accumulation of the nanoparticles *in vivo*.

Keywords: Polylactic acid; Nanoparticles; Multifunctionalization; Brain tumor; Target.

INTRODUCTION

The ultimate goal of cancer therapy is to selectively destroy cancer cells while sparing normal tissues^[1]. In this respect, site and cancer cell-specific drug delivery systems play a crucial role in achieving substantial tumoricidal effects due to the anticancer agents as well as in minimizing systemic side effects^[2]. The site-specific drug delivery may be achieved by localized implanting drug-carried wafer^[3], stereotactic injection^[4] or passive targeting delivery by microspheres^[5]. But the most promising way seems to be the active targeting delivery of drug by surface-functionalized nanoparticles^[6]. By chemical coupling or physical coating information-carrying ligands such as proteins or carbohydrates on the surface, the nanoparticles can be specially recognized and endocytosed by cancer cells. So far, most reports deal with monofunctionalized nanoparticles, *i.e.*, only one type of ligand that has specific affinity to receptors of tumor cells was coupled onto the surface^[7]. This strategy is workable *in vitro* cell experiments. *In vivo*, however, the performance of these nanoparticles could be hampered by biological barriers. Biological barriers, such as blood-brain-barrier (BBB), hinder the access of drug-loaded nanoparticles to tumor cells, rendering the tumor targeted drug delivery by these nanoparticles inappropriate even though tumor-specific ligand was conjugated on the surface. A potential protocol may be assembled of multiple functional factors on the nanoparticles surface, that is, mediate transport of nanoparticles across the biological barrier by one factor and bring the nanoparticles targeted to tumor cells by another. With this approach,

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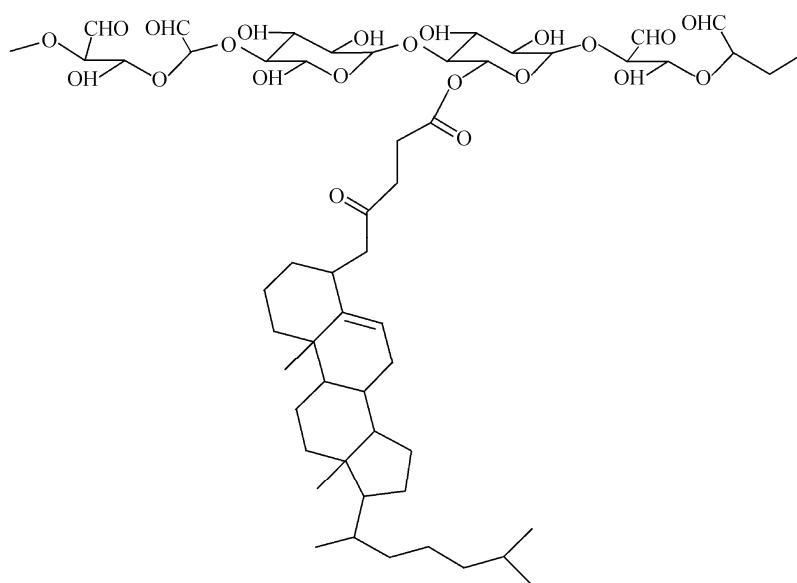
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epidermal growth factor receptor RNA interference genes have been successfully delivered to brain glioma by immunoliposomes functionalized simultaneously with two types of monoclonal antibodies targeting to human insulin and transferrin receptor^[8], respectively. To our knowledge, however, no study in the literature deals with multi-ligands functionalization of biodegradable poly(lactic acid) (PLA) nanoparticles.

PLA nanoparticles has been found to be an attractive drug carrier due to their non-toxicity, biocompatibility and biodegradability. One of the advantages for these drug delivery systems is the sustained release of drug, which prolongs tumor exposure to cytotoxic concentrations of chemotherapeutant, and improves the therapeutic efficacy. However, the surface functionalization of PLA nanoparticles is still a challenge because of the absence of reactive groups on the backbone. Recently, a novel method was developed to functionalize the PLA nanoparticles, *i.e.*, attached biofactors to the surface of the nanoparticles mediated by amphiphilic dextran dialdehyde coating^[9]. The aim of this study was to conceive of assembly of multiple biofunctional ligands on the surface of PLA nanoparticles based on this polysaccharide coating technology. To achieve this goal, cholesterol hydrophobically-modified dextran polyaldehyde (Chol-Dex-CHO) was synthesized (structure shown in Scheme 1), and dextran surface-decorated PLA nanoparticles bearing aldehyde groups were prepared through co-dialyzed DMSO solution of Chol-Dex-CHO and PLA. The resultant nanoparticles were then functionalized on the surface with two bioactive ligands, transferrin (Tf) monoclonal antibody and EGFR polyclonal antibody (as show in Scheme 2). The difunctionalized nanoparticles radiolabelled with ^{99m}Tc were administered using an In Situ Rat Brain Perfusion Technoique^[10], the targeting ability of the nanoparticles to the glioma was evaluated by using the scintillation counter.



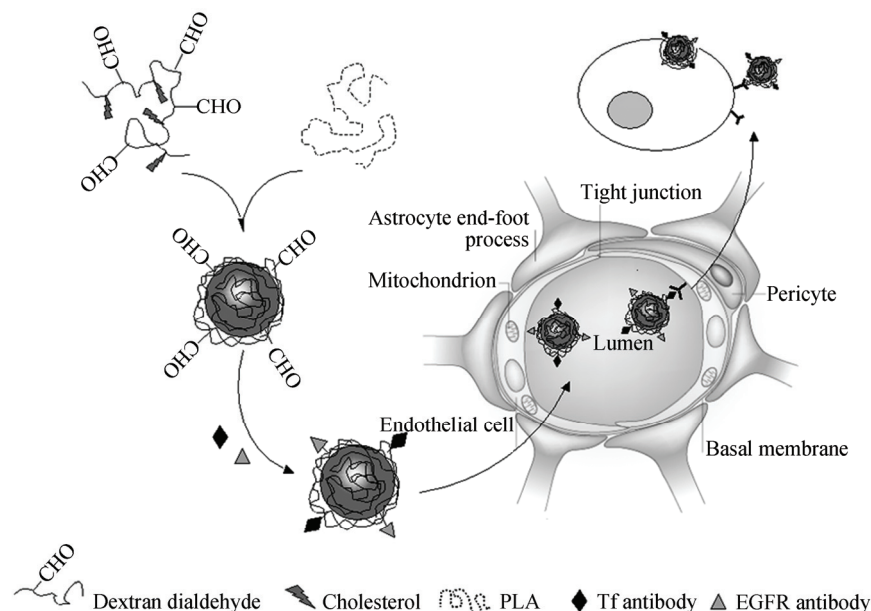
Scheme 1 Structure of cholesterol modified dextran polyaldehyde

EXPERIMENTS

Reagents and Materials

PLA ($M_w = 30$ kD) was purchased from Sigma while Dextran ($M_n = 40$ kD) was from Amersham Biosciences (Uppsala, Sweden). Cholesterol was obtained from Tianjin Chemical Reagent Co, China, and recrystallized in ethanol before use. CD71 and EGFR monoclonal antibodies were supplied by Santa Cruz Biotechnology Inc. Diethylenetriaminepentaacetic acid anhydride (DTPAA) was obtained from Fluka. Sodium pertechnetate ($\text{Na}^{99\text{m}}\text{TcO}_4$) was purchased from Shanghai GMS Pharmaceutical Co., Ltd. The water used in these studies was double distilled, and all other reagents and organic solvents were reagent grade or better. Sprague-Dawley rats

were purchased from Animal Center of the Chinese Military Medical Science Institute (Beijing, China). The Jiangwan animal stereotactic device model I C was from Shanghai Second Military University (Shanghai, China).



Scheme 2 Schematic representation of the assembly of multiple ligand onto surface of PLA nanoparticles, and the mechanism of the multifunctionalized nanoparticles targeting to glioma after transporting through blood-brain-barrier mediated by the transferrin-transferrin receptor recognition

Synthesis of Cholesterol Hydrophobically Modified Dextran Derivatives Containing Aldehyde Groups

Cholesterol modified dextran derivatives containing aldehyde groups (Chol-Dex-CHO) was synthesized by the method developed in the previous study^[9]. Briefly, cholesterol was esterified with succinic anhydride in anhydrous pyridine. The resulting cholesterol 3-hemisuccinate was then reacted with SOCl_2 in anhydrous chloroform to obtain cholesterol 3-hemisuccinyl chloride. DEX-CH was obtained by esterification of dextran polyaldehyde, which was obtained *via* oxidation of dextran by NaIO_4 , with cholesterol 3-hemisuccinyl chloride using anhydrous dimethyl sulfoxide (DMSO) as a solution and triethylamine as a catalyst. The structure of Chol-Dex-CHO was confirmed by $^1\text{H-NMR}$ ($\text{DMSO}-d_6$, Varian UMTY plus 400 NMR spectrometer). The substituted degree (SD), determined from $^1\text{H-NMR}$ spectrum and defined as percent content of cholesterol per 100 glucopyranosidic units, was controlled by varying the amount of cholesterol 3-hemisuccinyl chloride and the mole ratio of (Ugl) of dextran to cholesterol.

Nanoparticles Preparation and Characterization

The nanoparticles were prepared by a co-dialysis method. In detail, 8 mg PLA and 40 mg Chol-Dex-CHO was co-dissolved in 20 mL dimethyl sulphoxide (DMSO) with magnetic stirring. The resultant solution was dialyzed against deionized water under ice-bath for 72 h. Then the resulting suspension was centrifugated at 16000 r/min for 10 min and followed by washing three times with normal saline under sonication. PLA/Chol-Dex-CHO nanoparticles were finally obtained by freeze-drying.

Surface Functionalization of the Nanoparticles

The content of aldehyde groups on the PLA/Chol-Dex-CHO nanoparticles was determined as 0.052 mmol per milligram nanoparticles by Schales' method^[11].

CD71 and EGFR antibodies were added in the ratio of 1:1 (mol/mol), and the ratio of antibodies to aldehyde groups was 1.5:1 (mol/mol). The antibodies were incubated with nanoparticles overnight, and then 0.1 mL NaBH_4 (0.1 mol/L) were added to reduce the Schiff's base. The solution was submitted to centrifugation, and the

supernatants were collected. The antibodies remained in the solution were measured by ultraviolet spectrophotometry (measured at 280 nm).

To evaluate the effect of biofunctionalization on nanoparticle size, nanoparticle size analyses were carried out on a particle size analyzer (PSA, Brookhaven Instruments Corp. 90 Plus, USA). The nanoparticles were dispersed in physiological saline and sonicated for 2 min at 40 W, then their effective diameters were recorded at 90° scattering angle under 25°C without filtering.

The morphology of the PLA/Chol-Dex-CHO nanoparticles was observed by using a transmission electron microscope (TEM) (JEM-2000 FX II, Jeol, Japan). A drop of the nanoparticles suspension was placed on a copper grid coated with carbon film and dried at 25°C. Observation was performed at 80 kV.

Radio Labeling of Tf-PLA Nanoparticles

50 mg blank antibodies functionalized PLA nanoparticles were dispersed in 0.1 mol/L sodium phosphate, 0.1 mol/L NaCl buffer (pH 8.0), then 6 mg DTPAA was added in solid forms. The mixture was stirred for 60 min at room temperature, and then subjected to centrifugation at 16000 r/min for 10 min and followed by washing three times with buffer under sonication condition. The nanoparticles were resuspended in acetate buffer (0.1 mol/L, pH 5.6) by sonication, 0.3 mL Na^{99m}TcO₄ (2.2 mCi) in saline and 0.5 mL SnCl₂ in hydrochloric acid solution (0.01 mol/L) were added. The mixture was stirred gently and allowed to stand at room temperature for 10 min. The labeled nanoparticles were then purified by centrifugation and sonication, and washed with normal saline until the radioactivity of the nanoparticles measured by gamma ray spectrometer was stable. The labeling efficiency was expressed as a percentage of the total amount of radioactivity applied in the testing system.

Stability of ^{99m}Tc-labeled nanoparticles was determined *in vitro* in human serum by ascending thin layer chromatography (TLC) technique. The labeled nanoparticles (0.1 mL) were incubated with freshly collected human serum (0.4 mL) at 37°C for more than 8 h. The samples were withdrawn at regular intervals and spotted on instant thin layer chromatography strips, followed by development using acetone as the mobile phase. The solvent front was allowed to reach up to a height of *ca.* 5 cm from the origin. The strip was cut into 2 pieces, and the radioactivity in each piece was determined by a gamma ray spectrometer.

Animal Model

Adult male Sprague-Dawley rats weighing between 200–250 g were anesthetized by intraperitoneal injection with 10% chloral hydrate (300 mg/kg) and then fixed in a stereotactic apparatus. A 1.2 mm burr hole was drilled in the right side of the skull, 1 mm anterior and 3.0–3.5 mm lateral to bregma to expose the dura. Using a microliter syringe equipped with a 26-gauge needle and connected to the manipulating arm of the stereotactic apparatus, 1 × 10⁶ of C6 glioma cells in 10 µL serum-free DMEM were injected into the caudate nucleus at a depth of 4.0–4.5 mm from the dura over a 10 min period. The needle was left in place for 10 min and then slowly withdrawn. The burr hole was filled with bone wax and the scalp wound was closed with silk threads. Tumor formation was determined by MRI on the 5th day following injection.

Brain Target of Multifunctionalized Nanoparticles

To evaluate the brain target of the multifunctionalized of nanoparticles, monofunctionalized nanoparticles bearing CD71 or EGFR were prepared. The PLA nanoparticles coated with dextran were also prepared by co-dialysis of cholesterol modified dextran and PLA, and were used as control sample. After anaesthesia induced with an i.p. injection of a saline solution of xylazine (4.0 mg/kg) and ketamine (100 mg/kg), a catheter filled with heparinized saline (100 units/mL) was placed into the left common carotid artery after ligation of the left external carotid, occipital and common carotid arteries. The pterygopalatine artery was left open during the experiments. A suspension of Np (0.5 mL of suspension in saline containing 0.5 mg of Np each animal weighing 250 g) was perfused with 60 s followed by 120–180 s of perfusion with PBS solution. 2 h later, rats was sacrificed, and left and right brain, liver, spleen, kidney were excised, washed quickly with cold water to remove surface blood, and counted for radioactivity.

RESULTS AND DISCUSSION

Characterization of Cholesterol Hydrophobic-Modified Dextran Containing Aldehyde Groups

The aim of the present study was to assemble multiple ligands on PLA nanoparticles based on the polysaccharide surface decorating technology. The polysaccharide used needed modification by hydrophobic moieties which would be embedded inside the outer part of the PLA core, leading to the anchoring of polysaccharide on the surface of nanoparticles^[12]. Besides, the polysaccharide should provide high reactive active groups with the following attachments of ligands.

To achieve the above goal, cholesterol hydrophobic-modified dextran polyaldehyde was synthesized (Fig. 1). The oxidation degree of dextran, expressed in terms of the aldehyde group content per 100 glycosidic units of dextran determined by Schales' method, was controlled less than 10%. This oxidation degree might keep the feature of dextran protecting decorated nanoparticles against protein adsorption^[13], and meanwhile, supply sufficient aldehyde groups for the coupling of adipic acid dihydrazide and the attachment of biotin. The chemical structures of the conjugates were ascertained by ¹H-NMR spectroscopy (Fig. 2), and the substitution degree of cholesterol was estimated^[14]. By calculating the ratio integrated area for the related protons observed at $\delta = 0.59$ (CH₃ at position 18 of cholesterol) and $\delta = 4.90$ and $\delta = 4.83$ (anomeric protons of the dextran skeleton), the estimated substitution degree of cholesterol is 8%.

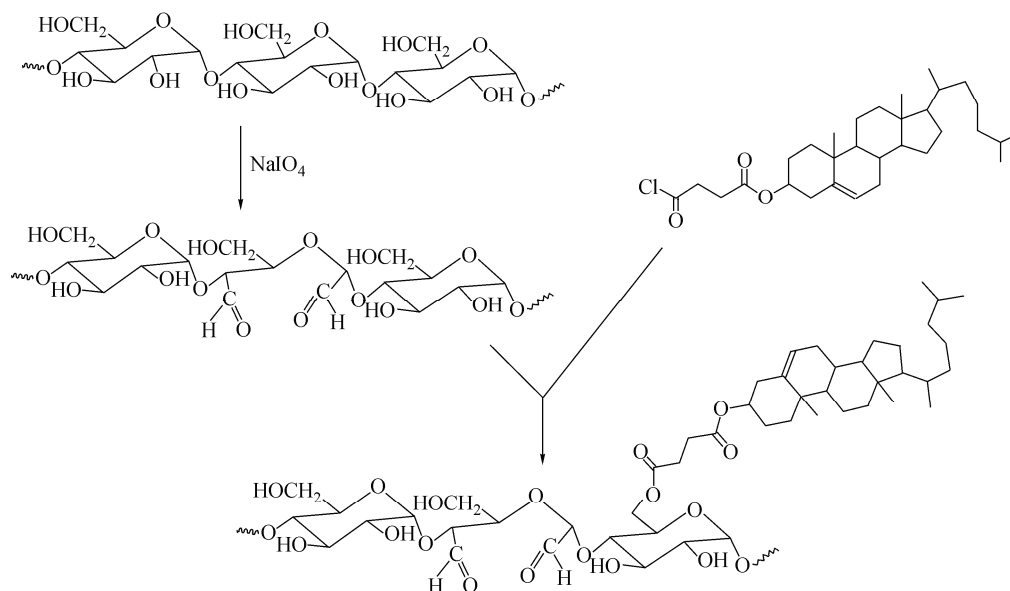


Fig. 1 Synthesis of cholesterol hydrophobic-modified dextran containing aldehyde groups

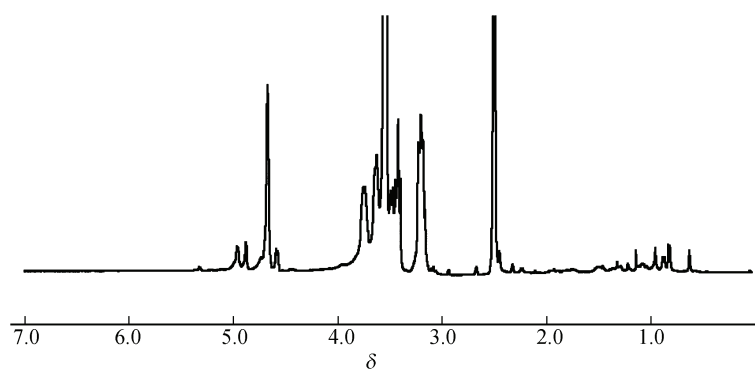


Fig. 2 ¹H-NMR spectrum of Chol-Dex-CHO in deuterated DMSO

Preparation and Characterization of PLA Nanoparticles Coated with Chol-Dex-CHO

The PLA nanoparticles bearing polysaccharides were prepared by co-dialysis the DMSO solution of PLA and Chol-Dex-CHO against distilled water. The morphology of the nanoparticles was observed by TEM, and the micrograph of a typical sample is shown in Fig. 3. The nanoparticles were well dispersed and spherical in shape. The size and size distribution of the nanoparticles were also measured by dynamic light scattering (Fig. 4). The average diameter of the nanoparticles was (160.8 ± 41.7) nm with a polydispersity index of 0.12, and this result coincides with that of TEM observations.

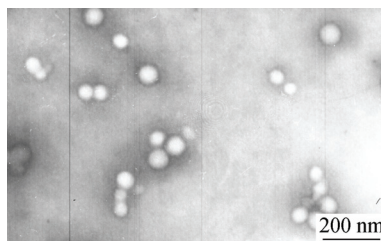


Fig. 3 TEM micrograph of PLA nanoparticles coated with Chol-Dex-CHO. The sample was negatively stained with phosphotungstic acid solution (20 g/L).

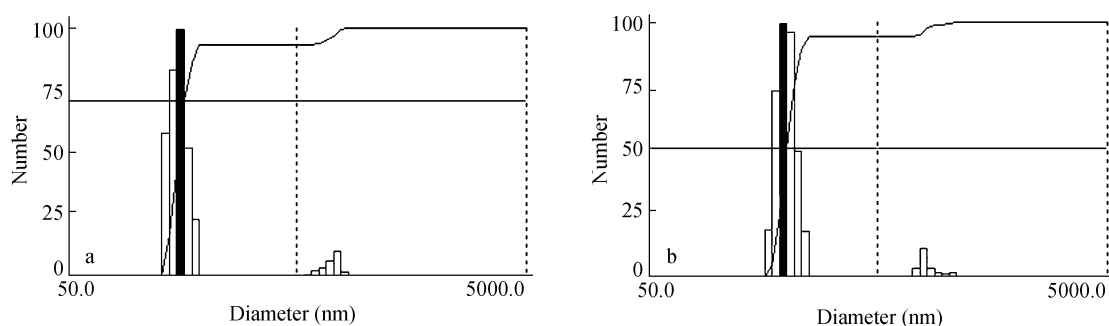


Fig. 4 Particle size distribution of the native PLA nanoparticles (a) and CD71 and EGFR multifunctionalized nanoparticles (b) measured by dynamic light scattering (DLS)

The small size, homogeneous size distribution and high assembly efficiency of dextran on the surface of nanoparticles should be ascribed to the advantage of diafiltration method. The dissolution of hydrophobic modified polysaccharide and PLA in co-solvent and then dialyzed against water facilitated the sufficient hydrophobic interaction between cholesterol pendent groups of the modified dextran and the PLA molecules, leading to the Langmuir type isotherm absorption of polysaccharide onto the surface of PLA nanoparticles^[12]. Furthermore, the hydrophobic modified polysaccharide can also act as a stabilizer, which prevents the PLA nanoparticles from aggregation in aqueous solutions. Indeed, the existence of polysaccharide is critical for the nanoparticles preparation by using diafiltration method. The polysaccharide-free dialysis of PLA nanoparticles led to occurrence of large lumps, and the yield of the nanoparticles was decreased dramatically. The diafiltration method also has the advantages of a low precipitation rate, high yield and one step procedure. The yield of the nanoparticles was over 85%, and the turbidity measurements suggested that the nanoparticles were kept stable at aqueous solutions for at least 4 weeks.

Surface Functionalization of Polysaccharide Coated PLA Nanoparticles

The ligand CD71 and EGFR antibody were coupled onto PLA nanoparticles through the Schiff's reaction between the amino groups of antibody and the aldehyde groups of oxidized dextran anchored on the nanoparticles through cholesterol pendants. The coupling of antibodies on the nanoparticles was proved by UV-

Vis measurements. Figure 5 shows the UV-Vis spectra of the functionalized nanoparticles. Compared with native particles, the functionalized nanoparticles exhibited a remarkable peak centered at $\lambda = 280$ nm, suggesting the existence of antibodies on the particles. The amount of the antibody coupled onto the nanoparticles was also estimated by UV-Vis analysis of the antibody amount left in the aqueous solution after functionalization reaction. The amount of antibody bound on the nanoparticles was 12 μ g per milligram nanoparticles.

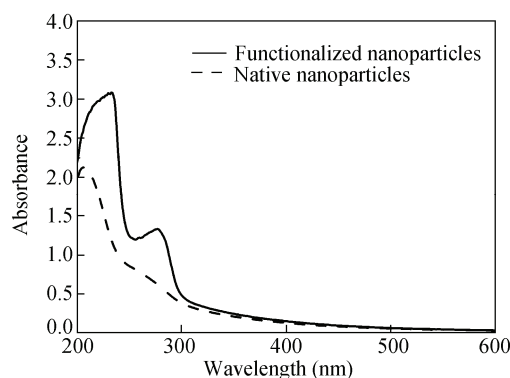


Fig. 5 UV-Vis spectra of functionalized PLA nanoparticles

Biodistribution and Targeting to Glioma in vivo

The biodistribution of radiolabeled multifunctionalized PLA nanoparticles in tumor bearing rats after 2 h of intracarotid perfusion is shown in Fig. 6. No matter how the nanoparticles were functionalized, they were always mainly uptaken by immune organs, *i.e.*, liver and spleen. The accumulation of the polysaccharide coated nanoparticles in the immune organs should be ascribed to the loops conformation of dextran which triggered the activation of both the alternative and classical pathways of the complement system, leading to the capture by the reticuloendothelial system^[15]. As a result, the accumulation of nanoparticles in brain is limited.

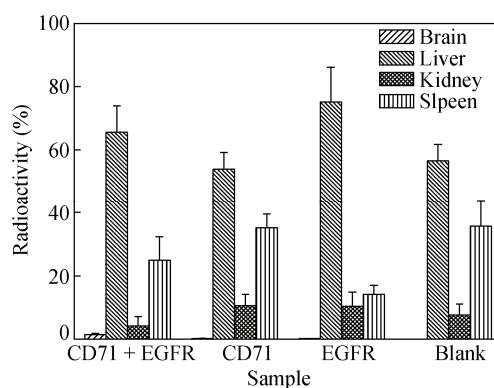


Fig. 6 Biodistribution of functionalized PLA nanoparticles in glioma tumor-bearing rats

The accumulation of nanoparticles in brain was evaluated by using relative ratio of radioactivity of brain to blood. As shown in Fig. 7(a), the relative radioactivity of virgin particles and the particles bearing EGFR antibody in brain post intravenous injection was found to be less than 1.0, indicating that few of these particles penetrated into brain. However, when CD71 were coupled on the surface of nanoparticles, the radioactivity in brain was obviously increased, suggesting that the existence of ligand corresponding to receptor expressed on the BBB is necessary for the penetration of the nanoparticles through BBB, even through the diameter of particles are larger than 100 nm. It has been believed that the size for the nanoparticles diffused across BBB should be less than 100 nm, but recent study proved that functionalized particles with the size larger than 200 nm could penetrate into BBB through the transcytosis mediated by the ligand-receptor interaction^[10]. Our results confirm

the early indications.

To determine the tumor targeting of the nanoparticles, the cerebra were separated into left and right sections and their radioactivity was measured, respectively. The brain tumor uptake of the nanoparticles was evaluated by using the relative percentage of radioactivity in cerebral hemisphere to that in total cerebra (Fig. 7b). It can be seen that there was no statistic difference in the radioactivity in the left and right cerebra for the native and EGFR antibody groups. In CD71 and CD71-EGFR groups, however, the radioactivity in right cerebrum (bearing tumor) was obviously higher than that in the left one, and more notably, multifunctionalized nanoparticles were found to be mainly accumulated in the right cerebral hemispheres. This result affirmed the hypothesis that the multifunctionalization will enhance the brain tumor targeting of the nanoparticles. The enhancement in glioma tumor uptake was also attributed to the multiplicate receptor overexpression in the tumor cells and tissues. In fact, both the receptor of transferrin and EGF are overexpression in the glioma tumor^[16–18], and the multifunctionalization of the nanoparticles might increase the possibility of the binding of nanoparticles to glioma cells.

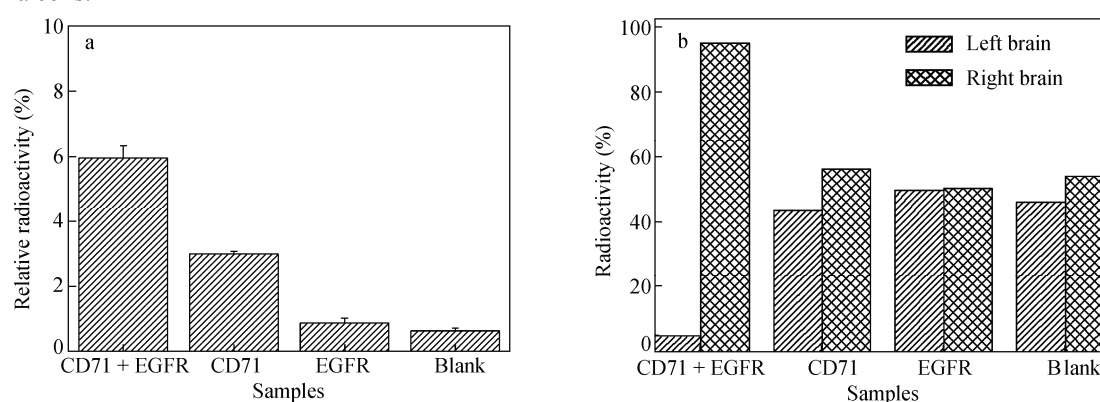


Fig. 7 Relative radioactivities of nanoparticles in brain (a) and in left and right cerebral hemispheres (b)

CONCLUSIONS

The polysaccharide coated PLA nanoparticles bearing aldehyde groups were prepared by dialysis of the DMSO solution of cholesterol hydrophobic-modified dextran polyaldehyde and PLA against water. The average diameter of the nanoparticles was about 160 nm, and the size distribution was nearly homogenous. The nanoparticles were functionalized simultaneously with CD71 and EGFR antibodies, radiolabeled with ^{99m}Tc, and then perfused into tumor-bearing rats through left common carotid artery. Multifunctionalized nanoparticles were found mainly to be accumulated in the RES system, and less than 2% of nanoparticles were uptaken by the brain. However, compared with monofunctionalized and native nanoparticles, the multifunctionalization enhanced the tumor accumulation of the nanoparticles *in vivo*.

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